GENETIC CHARACTERISATION OF THE COLOMBIAN PACIFIC COAST HUMPBACK WHALE POPULATION USING RAPD AND MITOCHONDRIAL DNA SEQUENCES

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Two genetic techniques were used to characterise the humpback whale population that overwinters annually off the Pacific Coast of Colombia. A preliminary study applied molecular techniques to an initial set of 32 biopsied or sloughed skin samples. Randomly Amplified Polymorphic DNA (RAPD) was used to provide an estimate of genetic variability and intra-population structure. Diversity of RAPD banding patterns suggest substantial genetic variability among sampled individuals. A parsimony tree was constructed using presence/absence of RAPD bands as characters, revealing three distinct groups: one of closely related individuals separate from two distinct clades within which relationships were unresolved. Mitochondrial DNA sequences for a consensus fragment 283 base pair in length of the rapidly evolving mitochondrial control region were then generated for the 32 samples and an additional 48 skin samples obtained from further fieldwork. An extensive comparative analysis was made with both published and unpublished control region sequences from humpback whales previously sampled in Colombia (n=64) and other regions in the Southern hemisphere (n=193) and the North Pacific (n=21). Haplotype diversity of the Colombian humpback population was high relative to other sampled populations, with 37 distinctive haplotypes, 11 of which were represented by a single animal. Both RAPD and mtDNA sequence data suggest further genetic substructure within the Colombian Pacific Coast humpback whale population. A large proportion of haplotypes (n=17) are shared with humpback whales sampled off the Antarctic Peninsula, suggesting a strong migratory connection between these regions as reported elsewhere. Only three haplotypes were shared with other Southern Hemisphere breeding grounds. Two Colombian haplotypes were common to populations from the North Pacific, supporting the hypothesis of a past or present East Pacific gene flow corridor between Northern and Southern Hemisphere populations.
❒ *Megaptera novaeangliae, population structure, RAPD, mitochondrial DNA.*

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The Colombian winter breeding ground, located between 2-3° north of the Equator off the Pacific Coast, has particular importance as a possible corridor of migratory overlap and genetic exchange between Northern and Southern Hemisphere humpback whale (*Megaptera novaeangliae*) populations of the eastern Pacific (Townsend, 1935; Flórez-González et al., 1998). Olavarría et al. (2000) suggested a migratory connection between overwinter sites off the Colombian Pacific coast and feeding grounds off the Antarctic Peninsula, based on photo-ID comparisons (Stone et al., 1990) and supported by mitochondrial data (Baker et al., 1998a). This research is one aspect of a long-term investigation of the Colombian Pacific Coast humpback whales by the Colombian non-governmental organisation Fundación Yubarta. In part, 144 skin

samples were obtained between 1991-1999 in two sampling locations (subregions), Gorgona Island and Málaga Bay (Fig. 1).

Whales are observed in this area from mid-June, peaking between August and October, until early December. This study reports the first genetic characterisation of this population using two molecular techniques, RAPD (Random Amplified Polymorphic DNA) patterns and comparative nucleic acid sequence analysis of a fragment of the mitochondrial control region (Dloop).

METHODS AND MATERIALS

DNA EXTRACTION. DNA was extracted from 144 skin samples obtained by biopsy darting or sloughed skin from 1991-1999. For biopsy darting, a small dart was fitted to an arrow (Lambersten, 1987). Sloughed skin was collected using a small nylon net (Amos et al., 1992). Three extraction protocols were used at different stages of the study: Sambrook et al. (1989); the 'QIAmp Tissue Mini Kit' protocol (Qiagen, Inc.); or the 'RapidPrep Micro Genomic DNA Isolation' (Amersham Pharmacia).

RAPD PROCEDURE. Four out of six short random primers (10 bp) were chosen for variable, reproducible banding patterns, after an initial screening. These primers were applied to an initial set of 32 Colombian humpback whale samples, as part of a preliminary study (Caballero, 1999). The primers were: P-1 (5'-GGTGCGGGAA-3'), P-3 (5'-GTAGA CCCGT-3'), P-4 (5'-AAGAGCCCGT-3'), P-6 (5'-CCCGTCAGCA-3') (Amersham Pharmacia Biotech). The PCR reaction mix 'Ready-to-go RAPD Analysis Kit' was used under the following low astringency amplification conditions: an initial denaturation cycle at 94°C for 2 minutes, 94°C for 1 minute, 62°C for 1 minute, 72°C for 2 minutes, 45 times. A final extension cycle was performed at 72°C for 5 minutes. PCR products were visualised in polyacrilamide gels stained with a silver solution. Dried gels were scanned and the migration rate (Rf) of each band was obtained. Comparing molecular weights of different bands (50 total) for each individual, a 0-1 matrix (presence-absence) was built. An outgroup set included two species of dolphins (*Tursiops truncatus* and *Stenella coeruleoalba*), two artiodactyl species, hippopotamus (*Hippopotamus amphibius*) and bull (*Bos taurus*) and a sample of human (*Homo sapiens*). Using this matrix, we calculated Jaccard's Genetic Distance

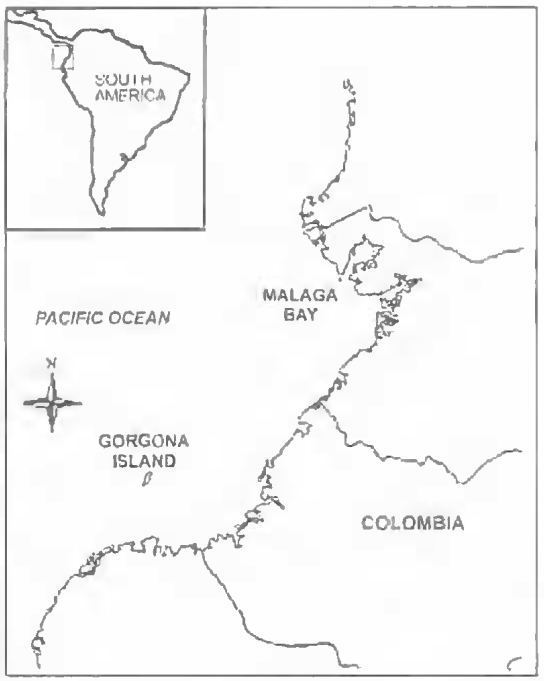

FIG. 1. Colombian winter breeding grounds, showing the two sampling subregions, Gorgona Island and Málaga Bay.

Coefficients by the UPGMA algorithm (Li, 1997) with SYNTAX 5 software. Jaccard's Genetic Distance was calculated as $D_{ij} = 1-[C/(2N-C)]$, where C is the number of common bands between individuals i and j, and N is the number of bands that are different in the two individuals. A consensus parsimony tree was built using the heuristic search option in PAUP version 4.02 software (Swofford, 1993).

Dloop SEQUENCES. For all 144 samples (the same 32 samples, and 48 additional samples), an ~ 550 base-pair fragment of the beginning of the mtDNA control region was amplified by PCR using standard reaction conditions (Saiki et al., 1988). Humpback whale samples from 1992 and 1996-99 field seasons were amplified with primers provided by R. LeDuc of the Southwest Fisheries Science Centre, La Jolla, California: TRO, a light strand primer, which spans portions of the tRNAs, threonine and proline, preceding the control region (5'-CCTCCCTAAAGAC TCAAGG-3'); and a heavy strand primer DH6, (5'-AAATACAYACAGGYCCAGCTA-3'). Samples from 1991 and 1995 field seasons were amplified using a different oligonucleotide primer pair that generated an overlapping

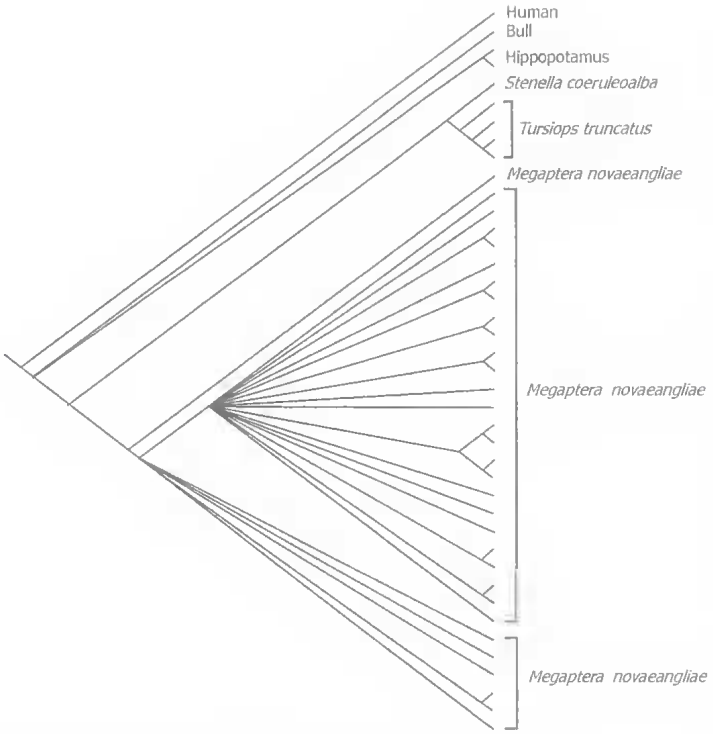

FIG. 2. Strict consensus tree from 194 trees of 263 steps.

fragment of the control region: light strand t-Pro-whale (5′-TCACCCAAAGCTGR ARTTCTA-3′), and heavy strand Dlp5 (5′CCATCGWGATGTCTTATTTAAGRGGAA -3′). Cleaned PCR products were sequenced on an ABI 377 automated sequencer using standard protocols of Big Dye™ terminator sequencing chemistry. Samples where sequenced in both directions to ensure homology of the obtained sequence. Sequences were reviewed and aligned using Sequencher 3.0 software (Genes Code Corporation). For comparative analysis with previously studied humpback whale populations from Colombia (n= 64), other Southern Hemisphere regions (n=193) and North Pacific (n=21), sequences were truncated to correspond with a 283 bp segment (Baker et al., 1993; Baker et al., 1998b). Identification of differences among sequences and determination of haplotypes was performed using McClade ver. 3.04.

RESULTS

Fifty molecular markers or bands, chosen for their reproducibility, obtained from the RAPD method were included in the analysis. Jaccard′s Genetic Distance value, determined among individuals of the species *Megaptera novaeangliae*, was approximately 0.56. This value, compared with those obtained between two delphinid species (0.58), can be interpreted as high for individuals of the same species (Caballero, 1999). As shown in the consensus tree (Fig. 2), the RAPD analysis of 32 individuals of *Megaptera novaeangliae* from Colombia identified three distinctive groups; one classifying possibly closely related individuals, the other two as unresolved groupings of individuals. Three other distinctive branches of the tree classified the delphinind species, clearly separated from the *Megaptera novaeangliae*, the artiodactyl species and the human sample as separate clades.

Mitochondrial control region sequence analysis revealed 37 haplotypes for the Colombian winter breeding ground. Sixteen were unique to this population (Table 1); 9 were shared with the Antarctic Peninsula feeding ground; 3 with other regions in the Southern Hemisphere; 7 with the Antarctic Peninsula and other regions in the Southern Hemisphere; and 2 with regions in the North Pacific, one of them shared also with the Antarctic Peninsula feeding ground (Caballero et al., 2000). The most common haplotype found in the Colombian winter breeding ground was shared with the Antarctic Peninsula but not with any other Southern Hemisphere location.

DISCUSSION

Randomly Amplified Polymorphic DNA (RAPD) is a molecular technique that has seldom been applied in studies of genetic variation in cetaceans. Analysis of RAPD markers among northern hemisphere minke whales (*Balaenoptera acutorostrata*) revealed the presence of two distinct stocks in the North Pacific and the North Atlantic, and the possible presence of only one breeding stock in the North Atlantic (Martinez & Pastene, 1999). Here we report the first use of RAPD analysis in humpback whales. These results indicate that a reasonable first approximation of population genetic variation may be obtained by application of the relatively fast and inexpensive method of RAPD analysis. These RAPD patterns

TABLE 1. Haplotypes of humpback whales sampled from Colombian winter breeding grounds detected among the 283 bp world-wide mtDNA Control Region consensus region sequences. • = same base as first sequence; – = presumed deletion; ? = unresolved; GI = Gorgona Island; BM = Málaga Bay.

MtDNA Haplotypes and polymorphic sites			No. of Indivuduals		
[10 20 30 40 50 60 70]			Subregion		
[. . . . . . .]			*GI*	*BM*	Total
GI9101	-CG--GCCT-C-AGCTCCACTTTTTTTAATAGTGCCCAATAGGTTCATTTGACTCTCTAGCTGCGACTCTCTC	[68]	13	21	34
GI9105	-..--...C-.-G.T.T.....A..T.....C.G.C.A.....AC.....C...	[68]	2	1	3
GI9110	-..--...C-.-....TT.....T.....T....A..C.....C.C...	[68]	3	4	7
GI9116	-..--....-.-..T.....C...CC...C.....C...	[68]	2		2
GI9117	-..--....-.-.A..T.....C...	[68]	4		4
GI9112	-..--....-.-G.T.TT.....T.....T....A...C.....C...	[68]	2	1	3
GI9124	-..--....-.-....T.....T.....T.....C...	[68]	2	6	8
GI9125	-..--....-.-..T.T.....CT.....C.....C...	[68]	2	2	4
GI9136	-..--....-.-....T.....A.C....C.....CT..	[68]	3	4	7
GI9130	-..--....-.-.A..T.....T.....C...	[68]	5	7	12
GI9142	-..--....-. ...T.....C...	[68]	1		1
GI9203	-..--...C-.-....TT.....T.....T....A..C.....CGC..??????	[68]	2		2
GI9209	-..--...C-.-....TT.....T.....T....A..C.....	[68]	2	8	10
GI9218	-..--....-.-.A..T.....CT.....C.....C...	[68]	1		1
BM9617	-..--....-.-.A..T.....CT.....C..G.....C...	[68]	2	7	9
BM9632	-..-T...C-.-....TT.....T....A..C.....C...	[69]		3	3
BM9634	-..--....-.-..T.T....C.....C....C.....C...	[68]		2	2
BM9614	-..--....-.-G.T.TT.....T.....T....A....C.....C.C...	[68]		4	4
BM9610	-..--....-.-.A..T.....T....A.....	[68]		1	1
BM9619	-..--....C.-..T.T.....T.....CT.....C...	[69]		2	2
BM9611	-..-T...C-.-....TT.....T.....T....A.....C...	[69]		3	3
BM9831	-..--....-.-.....T.....C....C.....CT..	[68]		2	2
BM9604	-..--....-.-....T.....C.....TT.....A.....	[68]		2	2
BM9537	-..--.T..-.-....T.....T....C..A.C....C.....CT..	[68]		3	3
BM9502	-..--....-.-..T.....T.....C...	[68]		2	2
BM9536	-..--....-.-..T.T.....T.....CT.....????	[68]		2	2
GI9106	-?.-T...C-.-....T.....T.....T....A.....C...	[69]	1		1
GI9107	-..--.T..-.-....T.....C.....T....C..A.C....C.....????????	[68]	1		1
GI9111	-?.--...C-.-G.T.T.....A..T.....C...C.A.....AC.....C...	[68]	1		1
GI9132	-?.--....-.-..T.TT.....T.....C.....A....C.....C.C...	[68]	1		1
GI9137	-..--....-.-.....A.C....C.....????????	[68]	1		1
GI9211	-..--....-.-..T.TT.....TT.....T....A.....G..???????	[68]	1		1
GI9217	-..--....-.-..T.....C.....C....C.....C...	[68]	1		1
GI9222	-..--....-.-..T.TT.....T.....T....A....C.....C.C...	[68]	1		1
BM9637	-..--....-.-..T.TT.....TT.....T....A.....C.C...	[68]		1	1
BM9823	-..--.T..-G-....T.....T....C..A.C....C.....G..G.GCTG.	[68]		1	1
BM9833	-..-T...-.-.....	[69]		1	1
Total Number of sampled individuals			144	144	144

suggest three groupings among Colombian humpback whales. These groupings could indicate further genetic sub-structuring on the Colombian wintering grounds, perhaps as a result of 'hidden stock' structure (Baker et al., 1998b).

Half of the mtDNA haplotypes identified in Colombia are shared with animals that spend austral summers feeding off the Antarctic Peninsula. The migratory connection between these two locations is thus highly supported, as established by Caballero et al. (2000) and Olavarría et al. (2000). The large number of shared haplotypes suggests this connection extends to the historical past and supports the migratory site fidelity hypothesis proposed by Baker et al., (1990).

The Pacific Coast of Colombia may be the point of transition for trans-hemisphere migration events as suggested by the presence of two haplotypes found in Colombia that are shared with animals sampled in the Pacific as far north as California and Japan. As the only documented overwintering site for Southern Hemisphere humpback whales located north of

the Equator, Colombian waters are uniquely situated to promote migration or genetic exchange between the northern and south-eastern Pacific. These shared haplotypes provide evidence to support the 'genetic corridor' hypothesis, which suggests past and/or present migratory connections between the eastern South Pacific and North Pacific humpback whale populations.

Combining the results of the two molecular techniques, we conclude that humpback whales that overwinter off Colombia's Pacific Coast represent a unique and diverse breeding population. Genetic characterisation of this population describes a group of animals represented by diverse maternal lineages, with high present day fidelity to this breeding site, but clear evidence of historical gene flow from other South and North Pacific Ocean stocks.

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